

# Genetic diversity and structure in the rare Colorado endemic plant *Physaria bellii* Mulligan (Brassicaceae)

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**Abstract** *Physaria bellii* (Brassicaceae) is a rare, outcrossing perennial endemic to shale and sandstone outcrops along the Front Range of northern Colorado, USA. This species is locally abundant, but ranked G2/S2—imperiled because of threats to its habitat and a small number of populations—according to NatureServe’s standardized ranking system. Leaf tissue from ten populations was analyzed with ISSR (Inter-Simple Sequence Repeat) markers to discern the amount of genetic diversity and degree of population subdivision in *P. bellii*. Genetic diversity was moderate (0.22) and a moderately high degree of population structure was found ( $F_{ST}$  calculated using two algorithms ranged from 0.17 to 0.24). An AMOVA partitioned most of the variation among individuals within populations (76%), and the remainder among populations (24%). Results from a Principal Coordinates analysis were consistent with the geographic distribution of populations. A Mantel test of the correlation between genetic and geographic distances was highly significant ( $P < 0.001$ ). The pattern of variation thus appears to be

distributed along a gradient, and efforts to conserve this species should involve preserving enough populations so that gene flow between populations is not interrupted.

**Keywords** ISSR · *Physaria* · Naturally rare plant · Dominant markers

## Introduction

Plant rarity is a function of both a species’ abundance and the range it occupies (Gaston 1994). Rabinowitz (1981) outlined seven forms of rarity, each of which has a different combination of three factors: geographic range (wide or narrow), habitat specificity (high or low) and local population size (large or small). Plant species with small geographic ranges and high habitat specificity are classic endemics, and many are threatened or endangered. Often, rarity is equated with vulnerability, but this is not always the case (Mace and Kershaw 1997; Rosenzweig and Lomolino 1997). Naturally rare species are often locally adapted to stressful habitats many other plants find unsuitable (e.g. Mateu-Andres and Segarra-Moragues 2000; Pepper and Norwood 2001; Mattner et al. 2002; de Lange and Norton 2004; Segarra-Moragues et al. 2005). Kunin and Gaston (1997) refer to this level of specialization as the “rarity trap” where plants are so well adapted to a narrowly defined habitat that they are precluded from expanding into other habitats. *Physaria bellii* Mulligan (Bell’s Twinpod) is an example of this phenomenon.

*Physaria bellii* is a specialist in terms of its preferred habitat, and is restricted to sloping shale and sandstone washes of the Niobrara, Pierre, Lykins and Fountain/

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Ingleside formations between elevations of 1,580 and 1,760 m along the Front Range in Colorado, USA (Fig. 1; Spackman et al. 1997; Doyle et al. 2004). These geological formations have a patchy distribution, and as a result, *P. bellii* does as well. According to the standardized Natural Heritage Methodology of NatureServe (Morse 1986; Colorado Natural Heritage Program (CNHP) 2006), *P. bellii* has a ranking of G2/S2, since there are a limited number of populations, and because this species depends on a habitat which is itself potentially threatened (Spackman et al. 1997; Doyle et al. 2004). Table 1 lists additional ecological characteristics of the *P. bellii* populations in this study.

*Physaria bellii* is an herbaceous, diploid ( $2N = 8$ ), perennial member of the Brassicaceae. Previous work suggests *P. bellii* is an early successional species whose presence is tied to small erosional disturbances that keep its preferred habitat relatively free of competitors (Supples 2001). *Physaria bellii* forms rosettes of leaves and bears several to many inflorescences, which flower from April through May, and set seed around the middle of July. Mulligan's experiments (Mulligan 1966), as well as greenhouse crosses conducted as part of a related study (L. Kothera unpublished), showed this species to be self-incompatible. The fruit is a small (4–6 mm) inflated silique consisting of two valves, each of which can hold a maximum of two seeds.

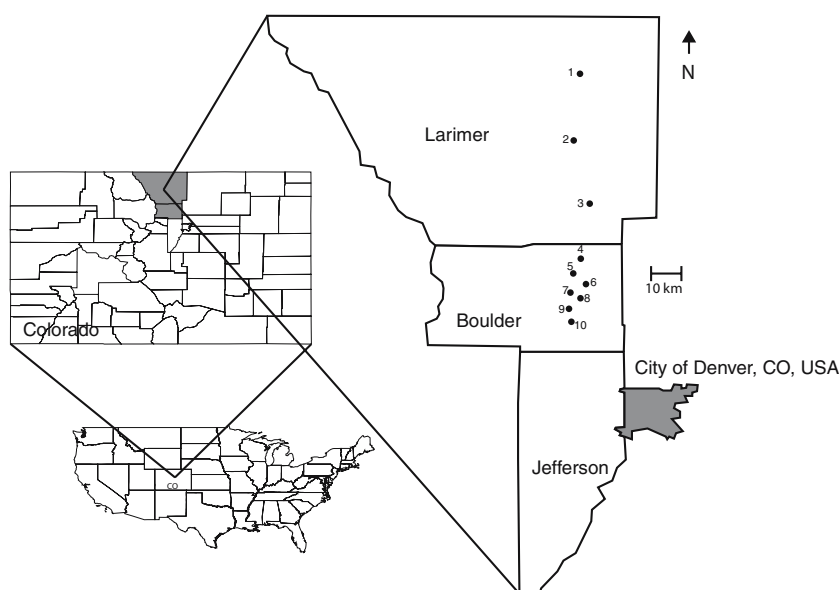
In contrast to species which have always been rare, recently rare species often occur as small, isolated populations in a formerly continuous habitat that has been fragmented (e.g. Llorens et al. 2004; Jacquemyn et al. 2004; Xiao et al. 2004; Hensen and Oberprieler 2005). Moreover, unlike many naturally rare species whose

populations are already discontinuous, populations of newly-rare species can have their viability compromised by the process of fragmentation (Frankham et al. 2002; Charlesworth 2003). Thus, while some rare species require intervention to remain viable, others need only periodic monitoring to ensure their continued persistence. For these reasons, naturally rare species with discontinuous distributions can serve as models to predict the genetic consequences of population subdivision in recently rare species (Fleishman et al. 2001).

Protocols that involve dominant genetic markers, such as RAPDs (Randomly Amplified Polymorphic DNA; Welsh and McClelland 1990; Williams et al. 1990), AFLPs (Amplified Fragment Length Polymorphism; Vos et al. 1995), and ISSRs (Inter-Simple Sequence Repeats; Gupta et al. 1994; Zietkiewicz et al. 1994) are widely used in the conservation of rare plants. ISSR markers were chosen for this study because their use is methodologically straightforward, and they have a higher consistency than RAPDs (Wolfe et al. 1998). As with all dominant markers, ISSRs are biallelic, meaning that a band is scored as either present or absent. Consequently, heterozygotes are not distinguishable from homozygous dominant individuals, which precludes a direct estimate of inbreeding.

This paper describes a population genetic study of *P. bellii*. We used a panel of ISSR markers to generate a series of complimentary measures to estimate the genetic diversity and the scale of genetic differentiation in this naturally rare endemic. The results are the first to describe population genetic parameters for this species, and will provide a valuable baseline for future management actions.

**Fig. 1** Map of *Physaria* populations used in this study



**Table 1** Ecological information and genetic diversity measures for populations of *Physaria bellii*

Population	Size	Substrate	Ownership	<i>h<sub>s</sub></i>	#Bands	<i>P</i>
1. North Poudre	Small	N	1	0.2526	30	48
2. Pine Ridge	Large	N	2	0.2643	34	55
3. Devil's Backbone	Small	FI	2	0.2288	28	45
4. Rabbit Mountain	Large	N	2	0.2530	31	50
5. Ryan	Small	N	2	0.2005	28	45
6. Steele	Large	N	2	0.1958	26	42
7. Lake Valley	Large	P	2	0.2180	26	42
8. Sage	Small	P	2	0.2298	27	44
9. City Boulder	Large	P	2	0.2317	28	45
10. Wonderland Lake	Small	P	2	0.2398	31	50

Small populations have <1,000 individuals, and Large populations have >1,000 individuals. Substrates: N, Niobrara shale; FI, Fountain/Ingeside sandstone; P, Pierre shale formations. Ownership: 1, private; 2, public; *h<sub>s</sub>*, Bayesian estimate of heterozygosity; *P*, percent polymorphic loci. Populations are listed from northernmost to southernmost

## Methods

### Sampling

Ten populations of *P. bellii* were sampled from along its entire geographic range (Larimer and Boulder counties; Fig. 1). The sample included some populations with less than 1,000 and some with more than 1,000 individuals, and included three substrate types (Table 1). At the time this study was initiated, there were approximately 20 known populations. Additional populations have since been found, bringing the known number of populations of *P. bellii* to approximately 28, all of which are periodically monitored by CNHP (Doyle et al. 2004). Two populations in Jefferson County were excluded because they were found to contain interspecific hybrids, based on intermediate leaf morphology (Kothera et al. Assessing the threat from hybridization to the rare Colorado endemic *Physaria bellii* in prep).

During the summer of 2002, plant tissue was collected for this and a companion study (Kothera et al. Assessing the threat from hybridization to the rare Colorado endemic *Physaria bellii* in prep) that examined the incidence of hybridization between *P. bellii* and the closely related *P. vitulifera*. The sampling method was designed to systematically collect representative, unbiased genetic diversity across this species'

range (Huenneke 1991; Owuor et al. 2003). A 300 m transect, which spanned most, if not all of the population, was started at the edge of each population and sampling commenced towards its interior. One individual was sampled at the beginning of each transect, and every 10 m thereafter. The sampled individual was chosen by flipping a coin to determine whether to walk left or right off of the transect, followed by rolling a six-sided die, which determined the number of steps taken before stopping to sample the closest individual. Sampling continued this way until 30 individuals from each population were collected. Leaf tissue was placed into individually numbered plastic bags and placed on ice until it could be stored at  $-80^{\circ}\text{C}$  pending subsequent DNA extraction.

### Molecular genotyping

Three “anchored” ISSR primers were used for this study. Each primer had one, two or three additional nucleotides on either the 3' or 5' end of the primer (Table 2). Using anchored ISSR primers is thought to increase their specificity by reducing the number of fragments of DNA that will bind with the primer, while still generating acceptable levels of polymorphism (Gupta et al. 1994; Zietkiewicz et al. 1994; Wolfe et al. 1998).

Genomic DNA was extracted from 100 mg of frozen leaf tissue using DNeasy Plant Mini Kits (Qiagen, Valencia, California, USA) according to the manufacturer's protocol. The concentration of DNA was quantified using a fluorometer and diluted with AE buffer from the Qiagen kits to a concentration of 10 ng/μl. ISSR reactions were carried out in 25 μl volumes containing 16.2 μl HPLC water, 2.6 μl 10× *Hot-Taq* PCR buffer (GeneSys, Buffalo, NY, USA), 1.5 μl MgCl (25 mM), 0.5 μl dNTP mix (100 mM), 2 μl primer (10 mM), 0.2 μl *Hot-Taq* Polymerase (GeneSys, Buffalo, NY, USA) and 2 μl template DNA (10 ng/μl). Reactions were carried out on a 96-well plate in a Hybaid thermal cycler with an initial cycle of 95°C for 10 min to activate the *Hot-Taq* polymerase

**Table 2** ISSR primer information

Primer name	Sequence	Annealing temperature (°C)	Total bands	Number (Percent) Polymorphic Fragments
UBC890	VHV(GT) <sub>7</sub>	51	22	11 (50%)
UBC809	(AG) <sub>8</sub> T	47	16	11 (69%)
UBC841	(GA) <sub>8</sub> YC	48	24	17 (71%)

A total of 39 polymorphic loci were used. Nucleotide abbreviations: V=G,A, or C; H=A,T or C and Y=T or C

and an additional 2 min at 94°C. This was followed by 35 cycles of 94°C for 30 s, annealing temperature (Table 2) for 45 s and 72°C for 90 s. There was a final extension step at 72°C for 20 min.

Four percent denatured polyacrylamide gels were used to resolve fragments. PCR product and formamide-based loading dye were heated in the thermal cycler at 95°C for 5 min to separate the DNA strands before loading the gel. A 100 bp DNA ladder was used with reference bands ranging from 100 to 1,500 bp (GeneSys, Buffalo, NY, USA). To minimize the effects of electrophoresis and staining on band variability, individuals from each population were divided into two groups and run on separate gels, thus any anomalies seen in one run and not the other could be investigated. In addition, some individuals' DNA underwent a second round of PCR and subsequent electrophoresis, which was then compared to previous runs to ensure that bands were being produced consistently. In every case, banding patterns on the first and second gels were identical. Gels were silver stained according to the method of Bassam and Caetano-Anollés (1993), allowed to air dry and scanned to create a permanent record.

#### Data analysis

The ISSR banding pattern of each individual was scored as the presence or absence of every polymorphic band, resulting in a data matrix of 1's and 0's for each of the 300 individuals. Only bands that could be scored consistently among populations were used.

The measures of genetic diversity calculated for this study included a Bayesian estimate of heterozygosity,  $h_s$ , determined using Hickory (version 1.0; Holsinger and Lewis 2003), which uses Markov's chain Monte Carlo (MCMC) simulations to produce posterior distributions based on the data. The use of  $h_s$  requires comparatively fewer assumptions regarding the data and provides a relative estimate of the genetic diversity, which allows comparisons across populations (Holsinger et al. 2002; Holsinger and Lewis 2003). In addition, the percentage of polymorphic loci,  $P$ , among all ISSR fragments was calculated.

An Analysis of Molecular Variance (AMOVA) was estimated using Arlequin (Version 3.0; Excoffier et al. 2005) to examine how genetic variation was distributed within and among populations. Statistical significance of the proportion of variance associated with the fixation index  $F_{ST}$  was determined with permutation tests against a null distribution generated by the data. The AMOVA procedure in Arlequin also provided estimates of pairwise  $F_{ST}$  values.

A measure of linkage disequilibrium (LD) across the sampled loci using exact tests was obtained using Arlequin. LD is a nonrandom association between pairs of loci (Hartl and Clark 1997). The overall level of LD in *P. bellii* populations was assessed using a method similar to that used by Miyashita et al. (1999) and Tero et al. (2003), which compared the frequency of instances of LD to the number of possible combinations of loci to determine if significant LD was present.

A Bayesian analysis of genetic structure was performed in Hickory. The default parameters of the program were used, which included using uniform priors, a burn-in period of 5,000 iterations, a run of 25,000 iterations and a thinning factor of 5. Each model was run five times to ensure the Markov chain was converging, and to make certain the results were consistent. The results were then averaged for the purpose of model comparison. Further comparisons were made between models with similar results by comparing posterior distributions of the parameter  $\Theta^B$ , which is analogous to  $F_{ST}$ .

To visualize differences among populations, a Principal Coordinates Analysis (PCOA) of Nei's (1978) unbiased genetic distances between populations was performed using the program GenAlEx (Peakall and Smouse 2001). A graph of the first two principal coordinates was generated, which plotted each *P. bellii* population as a point. Also, pairwise  $F_{ST}$  values were used to examine the relationship between population differentiation ( $F_{ST}$ ) and geographic distance (SAS Procedure Reg; SAS Institute, Inc. 2003). Finally, a Mantel test (Mantel 1967) was performed, which correlated population-level genetic distance (Nei 1978) and geographic distance matrices between populations (Tools for Population Genetic Analysis (TFPGA); Miller 2000).

## Results

### Genetic variability within populations

Thirty-nine polymorphic markers were scored for this study. Table 2 details the sequence, annealing temperature, total number of bands and degree of polymorphism for each of the three ISSR primers. ISSR fragments ranged in size from 280 to 1,350 base pairs. It is notable that each of the 300 individuals in the study had a unique multilocus genotype. The majority of fragments were found in more than one population, but populations 2 and 8 had one unique allele each. Values

for genetic diversity measures are included in Table 1. Levels are moderate, with populations 2, 1 and 4 showing the greatest diversity, followed by the southern populations (8–10) and the geographically central populations (5–7). The populations in the middle of *P. bellii*'s range exhibited lower levels of diversity.

Linkage disequilibrium was examined at the population level by Arlequin. There were 15,210 possible combinations of pairs of loci when each population was considered separately (all pairwise combinations of 39 markers  $\times$  10 populations). Of this total, 732 pairs, or 4.8%, showed significant linkage at the 0.05 level. This is approximately what would be expected by chance. Therefore, the 39 markers can be considered as independent loci.

#### Genetic structure at different hierarchical levels

The AMOVA (Table 3) indicated that most (76.4%) of the molecular variation in *P. bellii* exists among individuals within populations, with lesser amounts among populations (23.6%). Permutation tests suggest that the overall  $F_{ST}$  was significantly different from the null distribution ( $F_{ST} = 0.24$ ,  $P = 0.00$ ).

The Deviance Information Criterion (DIC; Spiegelhalter et al. 2002) generated by each run of Hickory was used to compare models. Lower values indicate a better fit of the model to the data. Although similar DIC values, 1,494 vs. 1,502, were obtained for the full (where both  $\Theta^B$  and  $f$ , analogous to the inbreeding coefficient,  $F_{IS}$ , are calculated) and the  $f = 0$  (where only  $\Theta^B$  is calculated) models, the  $f = 0$  model was chosen (see Discussion).  $F_{ST}$  calculated using the ' $f = 0$ ' model was 0.169 (range = 0.142–0.198). The other available models produced higher DIC values, which precluded their use to best explain these data.

#### Multivariate analysis of between-population relationships

The first two principal coordinates explained 42 and 27% of the total variance, respectively and correspond

fairly well with the geographic distribution of populations (Fig. 2). One exception to this trend is population 6. Pairwise  $F_{ST}$  values between populations were significantly different from the null distribution ( $P < 0.0001$ ), and ranged from 0.108 to 0.361. Population 6 was associated with consistently high pairwise  $F_{ST}$  values. When pairwise  $F_{ST}$  values were plotted against geographic distances, the relationship was linear (one-way ANOVA  $F = 16.943$ ,  $P < 0.001$ ; Fig. 3). Mantel test results show a significant correlation between the genetic and geographic distances among populations ( $r^2 = 0.590$ ,  $P < 0.001$ ).

#### Discussion

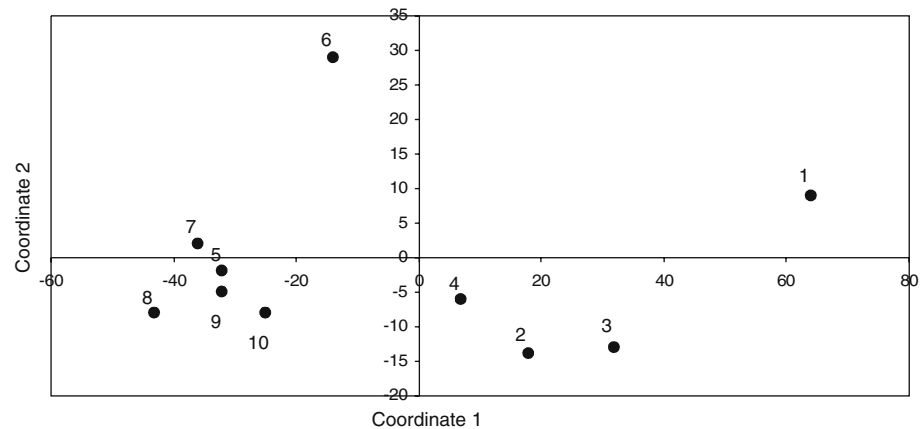
This study utilized several complimentary measures to describe the genetic diversity and degree of genetic differentiation in the endemic plant *P. bellii*. One set of measures (PCOA, Mantel Test) was distance-based. The other set of measures (AMOVA, calculations using Hickory) focused on population divergence based on approaches that partitioned heterozygosity. These measures were chosen because their use allowed the maximum amount of information to be obtained from a dominant marker data set. As a result, the ISSR analysis revealed that populations appear to be differentiated along a spatial gradient.

The AMOVA partitioned variation in *P. bellii* in a manner consistent with an outcrossing species with a patchy distribution. Thus, while most of the genetic variation (76.4%) existed within populations, some variation was also present among populations (23.6%; Table 3). Several other studies of outcrossing endemic species show similar patterns (Sales et al. 2001; Jacquemyn et al. 2004; Juan et al. 2004). Furthermore, permutation tests of the fixation index ( $F_{ST} = 0.24$ ) indicated significant genetic structuring. *Physaria bellii* populations show a strong north to south orientation (Fig. 1), which reflects the patchy distribution of the rocky substrates preferred by this species. Carpenter (1997) concluded that *P. bellii* is the dominant species in its preferred habitat, which is characterized by a high percentage of bare ground (Supples 2001). Noting a negative correlation between the presence of *P. bellii* and percent vegetation cover, Supples (2001) proposed a mechanism for the persistence of *P. bellii* in its preferred habitat. Because *P. bellii* is found significantly more often on sloped than on flat habitats, it may benefit from periodic erosional disturbances which remove or reduce competition (Donahue et al. 1983, cited by Supples 2001). The process by which favorable

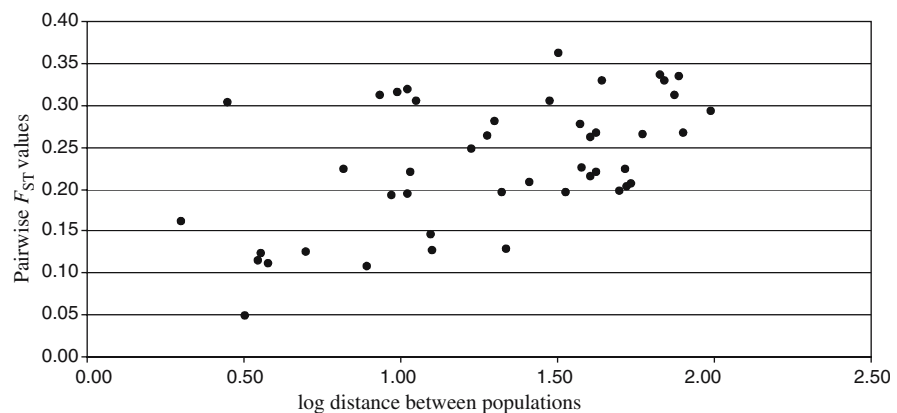
**Table 3** AMOVA results partitioning genetic variation within and among populations

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among populations	9	424.660	1.420	23.63
Within populations	290	1330.667	4.589	76.37
Total	299	1755.327	6.009	

**Fig. 2** Plot of the first two Principal Coordinates using Nei's (1978) genetic distance calculated among populations of *P. bellii*. The first and second Principal Coordinates explained 42% and 27% of the variance, respectively



**Fig. 3** Plot of pairwise  $F_{ST}$  values versus log distance between *P. bellii* populations



habitat is maintained is likely limited to the exposed rocky substrates of the above-mentioned geologic formations, which themselves display a patchy distribution. Thus, *P. bellii* would not be expected to expand its range under normal conditions.

Model comparison in Hickory reinforced the AMOVA results, and suggests that *P. bellii* populations are genetically differentiated. Estimating the inbreeding coefficient from dominant markers can be difficult or even unreliable (Lynch and Milligan 1994; Hardy 2003). For this reason, Hickory was used to obtain a qualitative measure of inbreeding only, in addition to an estimate of  $\Theta^B$ . The fact that two models produced similar DIC values has two implications (Holsinger and Lewis 2003). First, the small difference between the DIC values for the full versus the  $f = 0$  models (1,494 vs. 1,502) is at least consistent with low levels of inbreeding in *P. bellii*, as these results indicate no reason to choose the full over the  $f = 0$  model. Second, subsequent comparisons between the posterior distributions of  $\Theta^B$  for the two models also favored the  $f = 0$  model because the 95% credible interval for the difference in distributions did not contain zero. Thus, the

$f = 0$  model, where  $\Theta^B = 0.17$ , is preferable to the full model.

The graphical representation of inter-population relationships using PCOA (Fig. 2) is consistent with the geographic distribution of *P. bellii* populations. Notably, the southern populations, with exception of population 6, cluster together. Upon closer inspection, population 6 appears to be an outlier in this analysis. This view is supported by the presence of high pairwise  $F_{ST}$  values between this and all other *P. bellii* populations (range 0.30–0.36). Moreover, several outliers in Fig. 3 represent values from population 6.

The Mantel test revealed a significant correlation between the geographic and genetic distances among *P. bellii* populations ( $r^2 = 0.590$ ,  $P < 0.001$ ). Along with other evidence from this study, this finding suggests that the variation observed in this species is distributed along a north–south gradient. Populations of *P. bellii* are currently considered stable but may be vulnerable for two reasons. First, the linear distribution of populations suggests that the current scale of gene flow could be altered with the removal of even a few *P. bellii* populations. Accordingly, the critical

populations are probably those north of the city of Boulder, as they provide a conduit for gene flow between the northern and southern populations. Second, a recent survey by Doyle et al. (2004) noted that a significant number of occurrences of *P. bellii* are on private land, which is considered to be of high value for residential development and limestone mining. Populations could be extirpated if *P. bellii* habitat is used for other purposes, because the species lacks formal protection. Thus, continued monitoring of *P. bellii* populations, which should evaluate demographic characters as well as periodically estimate levels of genetic diversity and divergence, would most likely benefit this species. Furthermore, on-going educational efforts by local government open lands programs will help inform the public of threats to *P. bellii*. In this way, informed management decisions can be made that minimize the loss of diversity in this vulnerable species.

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